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Role of SMARCAD1 in human breast cancer cell proliferation, colony growth, migration and invasion

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A thesis submitted in partial fulfillment of the requirements for the

Degree of Master of Medical Science

In

Applied Pharmacology and Toxicology

Supervised by:
Dr. Samir Attoub

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Master's Thesis of Elham Mahmoud Al-Kubaisy **ID: 201080044**

Title of the Thesis: "Role of SMARCAD1 in human breast cancer cell proliferation, colony growth, migration and invasion"

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Dedication

This research thesis is lovingly dedicated to Dr. Samir Attoub, to our beloved teachers, and also to the students in the CMHS. In addition, most of all I dedicated this to my family, respective parents who have been my constant source of inspiration my father Prof. Mohmoud, my mother Wesal, my husband Ahmed Alshami he has given me the drive and discipline to tackle any task with enthusiasm and determination and friends for being a constant source of inspiration and support. Last but not the least; I dedicated this to GOD because without HIS love and support this project would not have been made possible.

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The guidance and support received from all the members who contributed and who are contributing to this project, was vital for the success of the project. I am grateful for their constant support and help.

Abstract

Breast cancer is the most common cancer seen in women worldwide accounting for more than 1.3 million cases and 458 000 deaths a year worldwide. Breast cancer patients are at high risk of recurrence in the form of metastatic disease. Genes associated with invasion and metastasis provides an aggressive edge in proliferation and growth during colonization of the metastatic site. The SMARCAD1 (SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1) protein is the human homolog of yeast Fun30 (Function Unknown Now 30) proteins. These proteins are members of the Snf2 helicase motif-containing protein families, and are known to act as ATP-dependent DNA translocators. Previous studies have shown that yeast Fun30 deletions are resistant to ultraviolet (UV) radiation. The over-expression of Fun30 has been shown to affect chromosome stability, integrity, and segregation. Fun30 has also been shown to be a potential cyclin-dependent kinase (Cdk1)/Cdc28 substrate. The SMARCAD1 is a DEAD/H box-containing helicase which includes proteins essential for replication, repair, and transcription. In addition to two DEAD/H box and an ATP-binding motifs within SMARCAD1, it has a putative nuclear localization signal and several regions that may mediate protein-protein interactions. Expression analysis indicates that SMARCAD1 transcripts are ubiquitous, with particularly high levels in endocrine tissue. The gene for SMARCAD1 has been mapped to the chromosome 4q22-q23m, a region rich in break points and deletion mutants of genes involved in several human diseases, notably soft tissue leiomyosarcoma, hepatocellular carcinoma, and hematologic malignancies. It has been recently reported that the binding sites of endogenous SMARCAD1/KIAA1122 are frequently found in the vicinity of transcriptional start sites. Moreover, human SMARCAD1 overexpression was observed in E1A-expressing cell line with increased capacity for gene reactivation events by genomic rearrangements suggesting that human SMARCAD1 may play a role in genetic instability. Previous studies in our laboratory showed that the breast cancer cells estrogen receptors (ER)-negative MDA-MB-231 and MDA-MB-231-1833 as well as ER-positive MCF-7 and T47D express a high level of SMARCAD1 in comparison with the normal breast epithelial cells NHME.

In my master research project, we investigated the impact of specific silencing of SMARCAD1 on human breast cancer cell proliferation, colony growth, morphology, migration and invasion using human breast cancer cells estrogen receptors (ER)-negative MDA-MB-231.

In this respect, the cells were stably transfected with two different designs of SMARTvector 2.0 Lentiviral shRNA particles targeting SMARCAD1. Control cells were transfected with SMARTvector 2.0 Non-Targeting control particles. The positive clones (10 to 12 from each design) were selected under puromycin and the GFP positive clones were analyzed using western-blot to confirm specific SMARCAD1 silencing. The two different design of shRNA targeting SMARCAD1 induced 99% decreased in the SMARCAD1 protein level (SMARCAD1-shRNA1, and SMARCAD1-shRNA3). The selectivity of this silencing was confirmed by the fact that no impact on SMARCAD1 protein was observed in the cells transfected with shRNA-control particles (control-shRNA). We demonstrated for the first time that silencing of SMARCAD1 resulted in a

significant inhibition of cellular proliferation and colonies formation in soft agar, as well as cellular migration, and invasiveness. All together, these results strongly suggest that SMARCAD1 may play an important role in breast cancer growth and metastasis.

Identification of new molecular effectors and signaling pathways involved in breast cancer growth, invasion, and metastasis could lead to effective targeted approaches in breast cancer therapy.

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Chapter 1

Introduction

1. Breast Cancer

Breast cancer is a tumor that forms in the mammary glands, most commonly from the inner lining of milk ducts or the lobules. It can affect both male and female; however, breast cancer in male, is rare.

2. Breast Cancer Epidemiology

2.1. Worldwide

Breast cancer is the second most common cancer in the world after lung cancer. It is however, the most common cancer worldwide in women with 1.38 million cases of breast cancer (23% of total cancer burden) in 2008. Breast cancer is the fifth cause of death from cancer overall (458 000 deaths), but it is still the most frequent cause of cancer death among women worldwide [1], [2]. One in nine women is estimated to develop breast cancer in her lifetime, and 1 in 29 is expected to die from it [3]. About 50% of the breast cancer cases and 60% of the deaths are expected to occur in developing countries. Western countries have the highest incidence rates, but lowest death rates. Some of the factors that added to the international variations in the incidence rates are largely the differences in reproductive and hormonal issues and the accessibility of early detection services. Recent use of postmenopausal hormone therapy or late age at first birth are considered reproductive factors that increase the risk of breast cancer. Alcohol consumption also increases the risk of breast cancer [2].

2.2. UAE

Cancer is the third leading cause of death in the UAE following cardiovascular diseases and accidents. Data from the UAE Ministry of Health point out that cancer accounts for approximately 500 deaths per year. Increasing

numbers of young women being detected with breast cancer, debunking earlier findings that women above 45 were more prone to the disease, according to HAAD's breast cancer awareness team. Breast cancer accounts for 28 per cent of all female deaths in the UAE. Previously women in their 40s and above were affected [4]. Unfortunately, over the past few years an increasing number of women in their early 30s and 20s were diagnosed with breast cancer due to the adoption of a Western lifestyle -- which primarily manifests itself in an increased intake of high-calorie food -- and lack of awareness on how to seek early treatment. Statistics show that 160 women develop breast cancer every year in Abu Dhabi alone. Breast cancer occurrence rates are very low among women below the age of 25. After this age, the incidence rate starts to rise. For example, roughly about one quarter of the breast cancers occur in the age group of 25 to 49 years, one quarter in women of 50 to 59 years of age, and the incidence peaks in women over 60 years of age – almost half of the cases fall into this age category. Even though the number of cases is increasing, the overall mortality due to breast cancer has decreased. Mostly, this is attributed to early detection and advanced treatment possibilities [3].

3. Breast Cancer Risk Factors

With regards to breast cancer, every woman is at risk of developing this disease. However, knowing the risk factors doesn't tell us everything. This does not mean that a female will get the disease by having a risk factor, or even several risk factors. Most women who have one or more breast cancer risk factors never develop the disease, while many women with breast cancer have no obvious risk factors (other than being a woman and growing older).

The most well recognized risk factors for breast cancer are age and family history. The risk of developing breast cancer increases by getting older and can be doubled every ten years, and at the age 70 breast cancer incidence can reach to 500 cases per 100,000 women [5]. About 1 out of 8 invasive breast cancers is established in women younger than 45, whereas about two out of three invasive breast cancers are found in women at age 55 or older [6]. However, young women diagnosed with breast cancer have more aggressive tumors associated with poor survival prognosis [7].

The main risk factor for developing breast cancer is simply being a woman. Gender is by far the most important risk factor. Breast cancer occurs 100 times more regularly in women than men [8]. The main reason why women develop more breast cancer is that women's breast cells are regularly exposed to the growth-promoting effects of the female hormones estrogen and progesterone [9].

Increase hormonal exposure in early menarche and late menopause is also considered as a risk factor. Early exposure to ionizing radiation, alcohol consumption, high breast density and some medications such as long-term postmenopausal hormone, like estrogen replacement are still controversial risk factor for breast cancer [10].

3.1. Hormonal factors

In postmenopausal women, elevated levels of some hormones, like estrogen and androgen, are associated with increased risk of breast cancer [11]. Females that have early menarche or late menopause have more menstrual cycles and this leads to longer exposure to estrogen and consequently to increase the risk of developing breast cancer. So any stimulus that leads to increase in exposure to these hormones is considered as a risk factor for breast cancer.

It has also been reported that women taking postmenopausal hormonal replacement therapy for five years or more have a high risk of developing breast cancer [12,13]. Several epidemiological and animal studies suggest that 17β-estradiol (E2) is directly involved in breast cancer *via* an unknown mechanism. Two pathways are involved in estrogen carcinogenesis: E2 metabolism and E2 estrogen receptor. 2-OH and 4-OH catechol estrogens are two metabolites of E2 that develop *via* phase I metabolism and oxidation. These metabolites cause DNA damage leading to cancer. In addition, *via* estrogen receptors, E2 can stimulate cell proliferation and gene expression [14].

3.2. Dietary factors

Several studies suggests an association between sunlight exposure and low breast cancer occurrence and mortality [15]. Vitamin D, which comes as a result of exposure to the sunlight, has anti carcinogenic properties [16]. The biologically active form of vitamin D, the 1,25(OH)2D, has anti-proliferative and pro-apoptotic effects and promotes the differentiation of breast cancer cells [15]. However, females with very dense breast tissue are at high risk of subsequent breast cancer [15].

Additionally, being obese increases the risk for developing breast cancer by 30 to 50%. Porter and his colleagues revealed that obese patients show larger, more advanced, and aggressive tumors [17]. This might be due to the increases in the production of estrogen through the activity of aromatase in breast adipose tissue [18]. The increases in breast cancer risk and mortality have been mainly associated with the upper body obesity as defined by the waist-to-hip ratio [19]. Increased estrogen production in obese patients is associated with the increased risk of breast cancer and enhanced progression of ER+ breast cancer [20]. Physical activity during adolescence may be particularly protective. However, minor differences were observed between physically active premenopausal women and sedentary women [21].

3.3. Lifestyle and Environmental factors

Smoking and alcohol consumption are also considered as risk factors. The growth of breast cancer cells could be enhanced by nicotine, which means that nicotine can be transported to the breast tissue via plasma lipoproteins following its passage through the alveolar membrane, and directly contribute to the molecular mechanism of breast carcinogenesis [22]. Comparing smokers to women who have never smoked or are not currently smoking but whose passive smoking exposure was unknown, the risk of breast cancer increased among the women who smoke cigarettes [23]. However, another study showed little or no association [24].

Women who are exposed to high doses of radiation, mostly through adolescence, have an increased risk of breast cancer. This link has been noticed among women who received high-dose radiation for medical purposes [25]. Uncertainties remain about the effects of low dose chest X-rays, particularly in individuals at increased genetic risk.

3.4. Genetic risk factors

Cancer is caused by either alterations in oncogenes, tumor-suppressor genes, and/or microRNA genes [26]. Around 5 to 10% of breast cancer cases are thought to be inherited resulting directly from gene defects (called mutations) inherited from a parent. The risk of breast cancer is increased two- to three-fold in female with a family history of breast cancer [27].

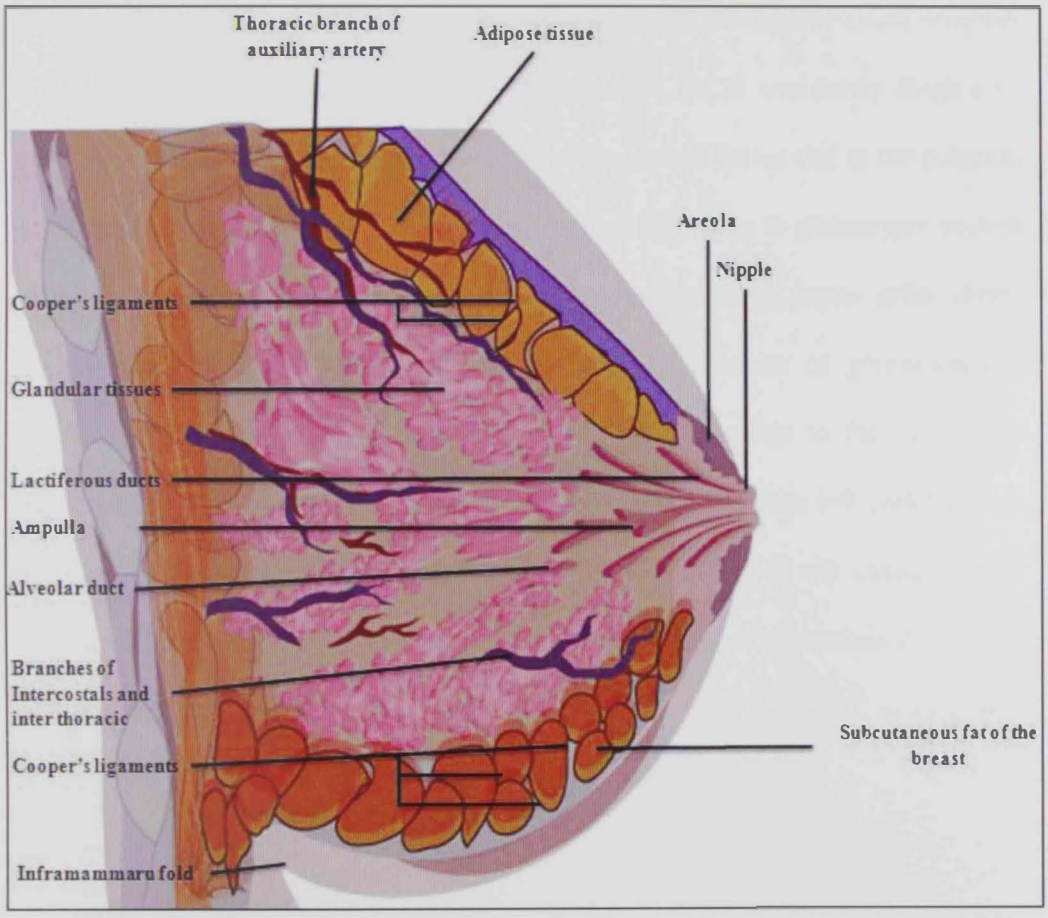
BRCA1 and BRCA2: In normal circumstances, expression of these genes helps to avoid cancer and prevent the cells from growing abnormally. Deletions and inactivating point mutations in these two genes cause a significant increase in the risk of developing breast cancer [28], [29], [6]. BRCA1 is located on chromosome 17 and play a role in many cellular process such as maintaining genome integrity, DNA repair and tumor transcriptional regulation and suppression. The percentage of women who develop breast cancer with mutation of BRCA1 is between 50-80% [30]. BRCA2 is located on chromosome 13 and it is responsible for preventing cells from uncontrolled proliferation and in repairing DNA damage. The risk of breast cancer in mutated BRCA2 is about 60-80% [27]. Family history of breast cancer is a well-known risk factor for breast cancer; the risk can increase in first-degree relatives with an average of about twofold [31]. In young women carrying gremlin mutations in *BRCA1* and *BRCA2*, breast cancer is likely to occur and often affects both breasts. Generally, tumors with *BRCA1* mutations are negative for estrogen and progesterone receptors. However, most *BRCA2* mutations are positive for these hormone receptors. Cells with mutations in *BRCA1* or *BRCA2* gene lack functional BRCA1 or BRCA2 protein and have a decreased ability to repair damaged DNA and grow in an uncontrolled manner [32].

P53 tumor suppressor gene: the first tumor suppressor gene identified was the p53 gene [33]. This gene is localized on the short arm of chromosome 17 and it encodes a 393-amino acid protein, which is expressed at very low levels in normal cells [34]. P53 remains the most frequently mutated gene in human cancers, about 50% of people with cancer have their P53 gene mutated, with an overall frequency of 25% in breast cancer patients [33], [35]. The most aggressive forms of breast cancer have P53 mutations, which are remarkably frequent, and apparently among the key driving factors [36]. The functions of p53 in normal circumstances are to inhibit the proliferation of abnormal cells, thus preventing neoplastic development. Activation of p53 signaling pathway, which used to be on 'standby' mode under normal cellular conditions, take place in response to cellular stresses, and several anticancer agents. The Li-Fraumeni syndrome can be caused by inherited mutations of the p53 and people with this syndrome have an increased risk of developing breast cancer, as well as several other cancers [29].

HER-2: The tyrosine kinase receptor HER-2 gene mediates critical signaling functions in normal and malignant breast epithelial cells. Approximately 20 to 25% of humans have an overexpression of HER-2 receptor, which can lead to breast cancer [37]. An aggressive clinical phenotype of breast cancer is related to overexpression of HER-2 that includes high-grade tumors, increased growth rates, early systemic metastasis, and decreased rates of overall survival. This HER-2 overexpression can result in changes in the biological features of breast cancer, including increased proliferation and motility, suppression of apoptosis, higher invasive and metastatic behavior, and independence of steroid hormones [37].

4. Types of breast cancer

The female mammary glands have a role in both nourishing the offspring as well as offering immunological protection to the newborn baby. Each breast has 15 to 20 lobes; each lobe has many smaller lobules, which end in dozens of little bulbs that can produce milk. Thin tubes called ducts are linked to lobes and lobules and eventually lead to the main lactiferous ducts, which open in the nipple (Schema 1). Fat, blood and lymphatic vessels occurring in each breast fill the spaces between the ducts and secretory units. The lymph vessels lead to small bean-shaped organs called lymph nodes, clusters of which are found in the axilla and in many other regions of the body.



Schema 1: Anatomy of the breast

Breast cancer can be classified into two types (ductal and lobular cancer) according to the structure of the breast affected by cancer. The most common type of breast cancer is ductal carcinoma (7 of every 10 women) followed by lobular carcinoma (1 of every 10 women).

Breast cancer is also classified according to its invasive status. If the cancer is judged to be non-invasive, it is called "*in situ*", and the invasive breast cancers are referred to as "infiltrating". The term "*in situ*" is defined as the cancer that has not spread into tissue other than the one it started in [38].

4.1. Ductal carcinoma *in situ* DCIS.

DCIS is a non-invasive breast cancer characterized by early-stage cell abnormalities in the breast ducts. If it is untreated, about 30-50% of DCIS progress to a more invasive types of cancer [39]. Before 1980, DCIS was rarely diagnosed, but now about 25 % of DCIS are diagnosed in the United States due to the progress in the diagnostic methods. DCIS can be classified according to phenotypic pattern of the cells (solid, cribriform, papillary, and micro-papillary), tumor grade (high, intermediate, and low grade), and the presence or absence of granulomatous mastitis [40]. Breast cancer deaths linked with DCIS are due to the subsequent development of invasive carcinoma or areas of invasion that were left undetected at the time of diagnosis. However, when properly treated, DCIS are cured. On the contrary, at least half of invasive carcinomas have developed metastasis at the time of diagnosis [8].

4.2. Infiltrating or invasive breast cancer.

This is an aggressive type of breast cancer, which metastasizes to different organs such as lungs and bones.

4.3. Inflammatory breast cancer (IBC).

IBC has a poor prognosis and presents with symptoms resembling an inflammation. The cancerous cell emboli block the lymph vessels, leading to rapid swelling of the breast, skin thickness and nipple retraction with redness and persistent itching [41]. In the United States, IBC represent up to 6% of breast cancer patients [42]. The 3-year survival rate for patients with IBC was far lower (34%) than that for patients with other types of breast cancer (90%) [43].

Stage Grading			
0	Tis	N0	M0
I	T1*	N0	M0
IIA	T0	N1	M0
	T1*	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Table 1: New TNM staging classification approved by AJCC [44]

5. Breast cancer stages

In order for the oncologist to design a treatment regimen for patients, cancer staging is important. In 2000, a breast cancer task force was constituted to advice American Joint Committee on cancer to receive the sixth edition of cancer staging manual [44]. With the new achievements in diagnosis and treatments in the field of breast cancer, changes were needed in the TNM-staging system (Table 1). The stage of breast cancer depends on the size of the breast tumor and whether it has spread to lymph nodes or other parts of the body. T refers to the primary tumor, N to the lymph nodes, and M to the existence or absence of metastasis in other organs of the body.

Stage 0: refers to the non-invasive breast cancer in which cancer cells have not spread into neighboring breast tissue or outside the duct in case of DCIS or outside lobule in the case of lobular carcinoma *in situ*.

Stage I: the breast tumor is no more than 2 cm in diameter and the cancer cells have not spread to the lymph nodes

Stage II: is divided into two subcategories IIA and IIB

Stage IIA: the tumor is no more than 2 cm in diameter and has spread to the auxiliary lymph nodes, or the tumor is between 2-5 cm in diameter and has not spread to the lymph nodes.

Stage IIB: the tumor is between 2 and 5 cm in diameter and has spread into the auxiliary lymph nodes or larger than 5 cm in diameter and has not spread to lymph nodes.

Stage III: is subdivided into three categories:

Stage IIIA occur in two main scenarios:

1. The tumor is no larger than 5 cm in diameter but has spread to auxiliary lymph nodes that grow together forming clumps. The cancer may also spread into the cervical lymph nodes.
2. The tumor is larger than 5 cm in diameter and the auxiliary lymph nodes do not adhere to each other or other tissues.

Stage IIIB: the tumor can be of any size and it spreads to the skin of the breast or chest wall. This stage may also include swelling of the breast and lumps in the skin of the breast so inflammatory breast cancer is considered at least stage IIIB.

Stage IIIC: the tumor can be of any size but it spreads to the lymph nodes area above or below the clavicles, chest wall and indoor the skin of breast.

Stage IV: it is known as distant metastatic cancer, the last stage of breast cancer, the tumor can be of any size, and cancer cells have spread to other parts of the body, such as the lungs, liver, bones, or brain. The 5year survival rate is 13% [8].

6. Breast cancer invasion and metastasis

Metastasis is a complex process, starting from the migration and invasion of cancer cells into the nearby tissues and lymphatic circulation. To form new colonies at metastatic sites, breast cancer cells must have access to the circulatory system and proliferate in vital organs, such as lungs, bones, brain and liver. In order to promote these steps of motility and invasion, the tumor microenvironment has an essential role, by either secretion of chemotactic factors, such as (chemokines, growth factors, and matrix metalloproteases, which are felt by cancer cell surface receptors, including integrins, receptor tyrosine kinases, and G protein-coupled receptors (GPCRs)) or by direct interactions with stromal cells [45].

Cellular motility/invasion in three-dimensional extracellular matrix (ECM) can be explained by the lamellipodium extension at the leading edge. This can lead to the formation of new focal adhesions complexes, secretion of surface proteases in contact with ECM and focal proteolysis. The cell body contraction by actomyosin complexes will end up by cytoplasmic tail detachment and cell movement. Breast cancer cells respond to chemotactic signals by reformation of their cytoskeleton through Rho family of small GTPases members Rho, Rac, and Cdc42. Rho controls the stress fibers and focal adhesion formation, Rac regulates membrane ruffling and lamellipodia, and Cdc42 regulates filopodia formation [45].

7. Pathogenesis of breast cancer: key genes and pathways

Familial breast cancer involved two human genes BRCA1 and BRCA2. The two BRCA genes serve as important regulators of cell-cycle "checkpoint control" mechanisms, leading to cell-cycle arrest and apoptosis, or to DNA repair. Germline mutations of BRCA1 and BRCA2 genes account for only 15-20% of breast cancer that clusters in families and less than 5% of breast cancer overall.

Sporadic breast cancers are characterize by abnormalities that have been identified in several genes including p53 and overexpression of several genes' products including HER-2 and estrogen.

7.1. Hormones and tyrosine kinase receptors (estrogen and HER-2 receptors)

It has long been recognized that estrogen is involved in the pathogenesis of breast cancer that expresses its receptor. In fact, at diagnosis, about 75% of breast cancer patients are positive for estrogen receptor (ER) expression, and the

remaining 25% of breast cancer patients are ER negative [46]. Distinct genes located on separate chromosomes (6 and 14, respectively) can produce two different ER: ERα and ERβ. The two receptors have different roles in breast development. These receptors are activated with estrogen binding and undergo some modifications, like dimerization and phosphorylation [47]. Increased ERα/ERβ ratio in breast cancer as compared with benign tumors and normal tissues suggest that ERα is mainly associated with breast cancer pathogenesis, while ERβ can protect against the mitogenic activity of estrogens in pre-malignant lesions [48]. Even though estrogen receptors are considered as weak prognostic factors, they are considered a strong predictive factors for identifying patients benefiting from endocrine/targeted therapy [49].

Epidermal growth factor receptors (EGFRs) are tyrosine kinase transmembrane receptors. EGFR family consists of four members: EGFR1 (ErbB1/HER1), EGFR2 (ErbB2/HER2), EGFR3 (ErbB3/HER3), and EGFR4 (ErbB4/HER4). These receptors share similar structures consisting of an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and an intra-cytoplasmic tyrosine kinase domain. HER-2/Neu oncogene is the most frequently amplified oncogene in breast cancer [50]. Furthermore, an aggressive phenotype of breast cancer with poor prognosis were associated with HER-2 over-expression in about 25 to 30% of breast tumors [51].

7.2. Non-hormonal factors (p53)

The tumor suppressor gene p53 was first identified in 1979 and located on chromosome 17 [52]. The p53 protein acts as a checkpoint for several stimuli that cause DNA damage and apoptosis such as heat shock, hypoxia, radiation and

chemical drugs. Once activated, it accumulates in the nucleus to increase the expression of mdm2 and p21WAF1/CIP1, leading to inhibition of cell proliferation through G1/G2 cell-cycle arrest [53-57]. This check point control, leads to the repair of DNA damage, or to apoptosis if the damage cannot be repaired [58]. The vast majority of mutations seen in p53 are missense mutations rather than truncations or deletions. While loss of the tumor-suppressive functions of this gene are well recognized, expression of mutant p53 is often seen at a high level in cancer and mutant p53 forms appear to be more stable than the wild-type tumor suppressor.

Mutations of p53 are seen in more than 50% of all cancers. One of the best known syndromes is Li-Fraumeni, in which mutation of p53 predisposes to a broad range of cancers, including breast cancer, early in life [59]. The p53 mutations occur in 30% of non-familial breast cancer patients [60]. It is considered the most frequently mutated gene in human breast cancer [61].

8. Diagnosis

Large scale mammography screenings beside women's awareness of the state of their breasts by palpation are the most important factors regarding the early detection of breast carcinoma and can result in decrease in mortality [62]. The American cancer society recommends an annual screening mammography for women who are more than 40 years old and at 25 years of age- with high risk of breast cancer, especially those with BRCA1 or BRCA2 mutations [62]. In the cases of family history of breast cancer, mammography should be performed at a younger age in order to ensure the detection of the onset of the carcinoma. Not only does the diagnosis need to be rapid, but also it is important that the correct subtype of the

breast cancer be defined. This is crucial for the treatment of choice and depends on the molecular profile of the cancer. Other significant factors concerning the treatment are the lymph node status and the tumor size [63].

After the initial suspicion of cancer based on mammography and/or palpation (such as dense mass in the radiograph, or a palpable lump), biopsy is often extracted from the site of the finding. Microscopic examination of the biopsy is the only way to confirm breast cancer. If cancer cells are present in the biopsy, further measures need to be undertaken, such as personal and family medical histories as well as general signs of health.

9. Current therapeutic approaches

Once a patient is diagnosed with breast cancer and the stage is determined, the most appropriate therapy should be determined. Breast cancer therapy consists of surgery to remove cancerous tissue, cytotoxic-chemotherapy, hormonal therapy, targeted therapy, and radiotherapy.

9.1. Chemotherapy

Chemotherapy can be referred to as cytotoxic drugs used to kill cancer cells. If the breast tumor is large, the lymph nodes are concerned or the tumor is found close to the chest wall muscles and also in the case of inflammatory breast cancer, chemotherapy might be recommended before surgery and sometimes even after the surgery.

Chemotherapy drugs can be classified into many groups according to how they work, their chemical structure, and their relationship to drugs.

Adjuvant chemotherapy is used to kill those hidden cancer cells after surgery.

Neoadjuvant chemotherapy: Giving chemotherapy first to shrink a large cancerous tumor, making it easier to be removed with surgery.

Several cytotoxic drugs are used in combinations to treat breast cancer. These include combinations of cyclophosphamide, 5-flurouracil, methotrexate, doxorubicin, docetaxel, and paclitaxel. Examples of combinations include: CMF, which consists of cyclophosphamide, methotrexate, and 5-flurouracil; FAC, which consists of 5-flurouracil, doxorubicin, and cyclophosphamide.

Chemotherapeutic drugs are classified into several groups and named depending on their cytotoxic mechanism of action, physiological behaviour, and source of the drug. Classifications include alkylating agents, anti-metabolites, anthracyclines, and microtubule stabilizers.

9.1.2. Alkylating agents.

They prevent the cancer cells from proliferation by directly damaging the DNA. These agents can work in all phases of the cell cycle. Alkylating agents are used as therapy in many different cancers, including leukemia as well as lung, ovarian, and breast cancer. Because these drugs damage the DNA, they can cause long-term damage to the bone marrow, and this can lead to acute leukemia, that is dose-dependent.

Cyclophosphamide: is a potent immunosuppressant agent. This prodrug should be oxidized to obtain the active metabolite ''phosphoramide mustard'' which exert its cytotoxic effect via transfer of the alkyl groups to the DNA leading to cell death [64].

9.1.3. Antimetabolites:

They inhibit purine and pyrimidine bases productions leading to abnormal DNA and RNA.

5-Fluorouracil: after its uptake by the cell, 5-FU is converted into different active metabolites, which is incorporated into RNA and produce aberrant mRNA species. As a pyrimidine analogue, it is also incorporated into DNA inducing inhibition of DNA synthesize and leading to cell cycle arrest and apoptosis [65,66].

9.1.4. Anthracyclines

Anthracyclines are anti-tumor antibiotics that were introduced in the 1960s. They exert their cytotoxic action through four major mechanisms including inhibition of topoisomerases II, blockage of DNA and RNA synthesis and DNA strand scission, binding to cellular membranes leading to the alteration of fluidity and ion transport and finally generation of free radicals through an enzyme-mediated reductive process [67].

Doxorubicin: FDA approved in 1989. It can be used as adjuvant or neoadjuvant chemotherapy to treat early stage of breast cancer as well as HER2+ breast cancer and metastatic breast cancer patients [68]. Doxorubicin can be used in several combinations such as with 5-flurouracil or cyclophosphamide.

9.1.5. Microtubule stabilizing agents. Include paclitaxel and docetaxel.

Paclitaxel (Taxol): is of a plant origin from Pacific Yew tree (Taxus brevifolia) found in North America [69]. Paclitaxel stabilizes microtubules and as a result, interferes with the normal breakdown of microtubules during cell division. Chromosomes are thus unable to achieve a metaphase spindle configuration. This blocks progression of mitosis, and prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell division [70]. It is recommended in advanced breast cancer.

9.2. Hormonal/Endocrine Therapy

For more than a century, endocrine therapies for breast cancer patients have been used. For breast cancer treatment, most endocrine therapies act as anti-proliferative of estradiol on oestrogen receptor (ER)-positive tumor in three ways; either estradiol is inhibited from binding to ER by anti-estrogens or reduction of estradiol concentrations by ovarian ablation in premenopausal women or by inhibition of aromatase in postmenopausal women to block estrogen production.

The first targeted breast cancer therapy was the anti-estrogen receptor Tamoxifen and because three quarters of breast cancer patients express estrogen and/or progesterone receptors, Tamoxifen is used as a first line therapy for the treatment of breast cancer. Even though it was found to be useful in treating estrogen positive breast cancer in both early and advanced cases, due to its partial estrogen agonist effect it had many side effects such as the risk of endometrial cancer [71,72].

Aromatase inhibitors are a class of endocrine/hormonal therapy. They act by inhibiting the cytochrome P-450 dependent enzyme aromatase, which is responsible for converting adrenal androgen substrates to estrogen. It is considered the only source of endogenous estrogen in postmenopausal women [73]. Aromatase inhibitors are divided into two groups: reversible and irreversible inhibitors. Reversible inhibitors such as Arimidex will block the enzyme for a limited time.

The second-line therapy after failure of Tamoxifen is Fulvestrant (selective oestrogen receptor down-regulator) and also a competitor with estrogen for binding to the estrogen receptors [74]. It is also used for the third-line therapy after failure of tamoxifen and aromatase [75].

9.3. HER-2 Receptor Targeted Therapy

Almost 30% of breast cancer patients are presented with Her-2 protein over-expression due to the amplification of Her-2 gene [76]. Her-2 overexpressing breast cancer is known for its aggressive nature and short disease-free periods [50,77,78]. Trastuzumab, a recombinant humanized monoclonal antibody targeting Her-2 receptor, proved its efficacy in the treatment of breast cancer. Trastuzumab targets the extracellular domain of HER-2 receptor leading to blockade of several signaling pathway such as phosphatidylinositol 3-kinase and mitogen-activated protein kinase and consequently to cell cycle arrest and apoptosis [79]. Due to its severe cardiotoxicity, the use of trastuzumab draws a big question towards its safety for patients.

10. Aims of the Research

Breast cancer is one of the most common malignancies among women. Genomic studies have increased our understanding of the breast cancer disease, and in combination with screening programs, it has improved survival outcomes of breast cancer patients. Remarkable progress has been made in the treatment of breast cancer over the years, such as hormonal therapy, which depends on tumor sensitivity to endocrine manipulation, as well as some targeted therapies that can be used selectively in those patients most likely to benefit. However, despite these advances, controversies remain, patients die, and a cure remains elusive.

The SMARCAD1 (SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1) protein is the human ortholog of yeast *Saccharomyce cerevisiae* Fun30 (Function Unknown Now 30) protein [80,81]. The Fun30 protein is a member of the Snf2 helicase motif-containing protein families, which are known to act as ATP-dependent DNA transclocators [82,83]. Previous studies have shown that yeast Fun30 deletions are resistant to ultraviolet (UV) radiation [84, 85]. The over-expression of Fun30 has been shown to affect chromosome stability, integrity, and segregation [85]. Similarly, Fft2, the *Saccharomyces pombe* homologue of Fun30, was found to play a role in maintaining chromosomal stability and for the maintenance of the chromatin structure required for kinetochore assembly [86]. In addition, Fun30 was shown to interact genetically with Cse4 (the histone variant at centromeres) and ChIP-seq data confirmed Fun30 binding to centromeres [87].

Fun30 has also been found to play a role in the promotion of the cell cycle along with origin recognition subunit ORC5. Double mutants of both Fun30 and OCR5 were found to accumulate in the G1 and early S phase using FACS analysis

[88]. ChIP-seq data shows that Fun30 is also located at Autonomously Replicating Sequences (ARS) suggesting a role of Fun30 in replication [87]. Fun30 has also been shown to be a potential cyclin-dependent kinase (Cdk1)/Cdc28 substrate [89].

As for its biological activity, it has recently been shown that purified Fun30 forms a homodimer of around 250 kDa and is able to hydrolyze ATP, bind to DNA, mononucleosomes and nucleosomal arrays. Fun30 was also shown to have a remodeling activity using restriction enzymes accessibility assay. In addition, Fun30 was found to be more efficient in exchanging histones dimers than in repositioning nucleosomes. These biochemical assays provided the first evidence that Fun30 is an ATP-dependent chromatin remodeler [81].

Other studies have also implicated Fun30 in heterochromatin regulation [88]; [86] and in DNA double strand break (DSB) repair by homologous recombination [90,91]. Fun30 was found to facilitate long-range DNA end resection in yeast cells and this required the ATPase activity of Fun30 and other resection proteins such as Sgs1 and Exo1. The authors suggested that the activity of Fun30 made the DNA more accessible to DNA repair machinery.

SMARCAD1, the human ortholog of Fun30, is a DEAD/H box-containing helicase, which includes proteins essential for replication, repair, and transcription. In addition to two DEAD/H box and an ATP-binding motifs within SMARCAD1, it has a putative nuclear localization signal and several regions that may mediate protein-protein interactions. Expression analysis indicates that SMARCAD1 transcripts are ubiquitous, with particularly high levels in endocrine tissue [92]. The gene for SMARCAD1 has been mapped to the chromosome 4q22-q23m, a region rich in break points and deletion mutants of genes involved in several human

diseases, notably soft tissue leiomyosarcoma, hepatocellular carcinoma, and hematologic malignancies [80,92]. It has been recently reported that the binding sites of endogenous SMARCAD1/KIAA1122 are frequently found in the vicinity of transcriptional start sites [93]. Moreover, human SMARCAD1 overexpression was observed in E1A-expressing cell line with increased capacity for gene reactivation events by genomic rearrangements suggesting that human SMARCAD1 may play a role in genetic instability development [92]. SMARCAD1 has also been shown to work at replication origin sites in order to facilitate deacetylation of newly assembled histones [94]. Interestingly, a recent paper suggested that SMARCAD1, just like Fun30, is involved in DNA end resection and was recruited to DNA damaged sites were H2A.X is phosphorylated. Silencing the SMARCAD1 gene affected the recruitment of Replication Protein A (RPA) indicating reduced formation of single-stranded DNA as in yeast cells [90]. These data together suggest an evolutionarily conserved role for the Fun30 and SMARCAD1 chromatin remodelers.

Dr. Attoub Laboratory have previously shown that the breast cancer cells estrogen receptors (ER)-negative MDA-MB-231 and MDA-MB-231-1833 as well as estrogen receptors (ER)-positive cells MCF-7 and T47D expressed a high level of SMARCAD1 protein in comparison with the human breast epithelial cells NHME.

My master's project aims to investigate the impact of specific silencing of SMARCAD1 on human breast cancer cell proliferation, colony growth in soft agar, morphology, migration and invasion using human breast cancer cells estrogen receptors (ER)-negative MDA-MB-231.

Chapter 2

Materials & Methods

2.1 Cell culture and reagents

Human breast cancer cells MDA-MB-231, MDA-MB-231-1833, MCF-7 and T47D were maintained in DMEM medium supplemented with antibiotics (penicillin 50U/ml; streptomycin 50µg/ml) (Invitrogen, Cergy Pontoise, France) and 10% fetal bovine serum (FBS, Biowest, Nouaille, France) at 37°C. The culture medium was changed every three days and cells were passed once a week when the culture reaches 95% confluence. The human breast epithelial cells NHME were maintained in MEBM™ plus SingleQuots™ of Growth Supplements (Lonza, Walkerville, MD, USA). In all experiments, cell viability was higher than 99% using trypan blue dye exclusion. Paclitaxel was purchased from Sigma-Aldrich (Sigma-Aldrich, Saint-Quentin Fallavier, France). Frondoside A of 99.9% purity was purified from Cucumaria frondosa, harvested near Stonington, Maine and checked by NMR as previously described [95,96]. SMARTvector 2.0 Lentiviral shRNA particles (Dharmacon Thermo Scientific, US) can directly bind to cells and deliver their genetically engineered RNA genome to the cytoplasm. The SMARTvector 2.0 includes a turboGFP reporter gene to facilitate assessment and optimization of transduction efficiencies. The SMARTvector 2.0 also contained a puromycin resistance gene for selection and isolation of clonal populations when generating stable cell lines.

2.2 Establishment of Stable SMARCAD1 silencing in breast cancer cells

MDA-MB-231 cells were seeded at a density of 20,000 cells / well into 96-well plates, in the presence of the serum and allowed to attach for 24h. Cells were transfected with SMARTvector 2.0 Lentiviral shRNA particles targeting SMARCAD1 or SMARTvector 2.0 Non-Targeting control particles (Dharmacon

Thermo Scientific, US). Selection of cells stably expressing SMARCAD1-shRNA and the Control-shRNA started 72 h post-transfection following the manufacturer's instructions (Dharmacon Thermo Scientific, US). Briefly, growth medium was aspirated from the cells and replaced with fresh selection medium containing 10 μg/mL of puromycin. Puromycin-containing medium was replaced every 2-3days with freshly prepared selection medium, and selection of stable cells expressing SMARCAD1-shRNA or Control-shRNA was completed in approximately 4 weeks from the beginning of selection. Multiple clones and pools of clones were expanded, harvested, and prepared for western blot analyses of SMARCAD1 expression.

2.3 Western blot analysis of SMARCAD1 protein expression

Total cellular proteins from stably transfected cells with SMARCAD1-shRNAs and control-shRNA were isolated using RIPA buffer (25Mm Tris.HCl pH 7.6, 1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.5% protease inhibitors cocktail (Sigma, Steinheim, Germany), 1% phenylmethylsulfonyl fluoride, and 1% phosphatase inhibitors cocktail (Thermo Scientific, Rockford, USA). The whole cell lysate was recovered by centrifugation at 14,000 rpm for 20 minutes at 4°C to remove insoluble material and 30-50 μg of proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel for SMARCAD1 expression. After electrophoresis, proteins were transferred on a nitro-cellulose membrane, blocked with 5% non-fat milk and probed with SMARCAD1 (Abcam, Bristol, UK, 1:500) and β-actin (Santa Cruz, California, USA, 1:1000) antibodies overnight at 4°C. The blot was washed, exposed to secondary antibodies and visualized using the ECL system (Perkin, Waltham, MA, US).

2.4 Cell proliferation assay

Stable MDA-MB-231 cells transfected with SMARCAD1-shRNAs (SMARCAD1-shRNA1 & SMARCAD1-shRNA3) or control sequences (Control-shRNA) were plated at the density of 50,000 cells into six-well tissue culture dishes supplemented with 10% FBS. Cancer cells were trypsinized, collected in 1 ml of medium and counted at an appropriate dilution every day for five consecutive days.

2.5 Soft-agar colony formation assay

A layer of agar containing 1 ml of 2.4% low melting temperature agar (Bio-Rad) dissolved in distilled water was poured into wells of a 6-well cell culture dish and allowed to set at 4°C for 5 minutes then incubated at 37°C for 30 min. A second layer (2.9 ml) containing 0.3% of low melting agar dissolved in growth media containing stably transfected cells with SMARCAD1-shRNAs or with control-shRNA (20×10^3 cells / ml) was placed on top of the first layer and allowed to set at 4°C for 5 minutes. After 30 minutes to 1-hour incubation in the humidified incubator at 37°C, growth medium (2 ml) was added on top of the second layer and the cells were incubated in a humidified incubator at 37°C for 3 weeks. Medium was changed twice a week. At the end of the experiment, colonies were stained for 1 hour with 2% Giemsa stain, and incubated with PBS overnight to remove excess stain. The colonies were photographed and scored.

2.6 Cellular Morphology

Cells were seeded on four-chamber culture slides at a density of 25 000 cells/chamber. After 48 h, chambers were removed; cells were washed with PBS, fixed in 4% paraformaldehyde for ten minutes at room temperature, washed with PBS and permeabilised with 0.1% Triton X-100 in PBS for 10 min. Then, the actin cytoskeleton was stained with rhodamine phalloidin (Invitrogen Molecular Probes, Oregon, US) and the nuclei were stained with 4', 6-diamine-2-phenylindole dihydrochloride (DAPI) (Invitrogen Molecular Probes, Oregon, USA). The cells were then photographed using an Olympus fluorescence microscope (Olympus 1X71, Japan) for the analysis of theirs morphology.

2.7 Wound healing motility assay

MDA-MB-231 cells stably transfected with SMARCAD1-shRNAs or control-shRNA were grown in six-well tissue culture dishes until confluence. Cultures were incubated for 10 min with Moscona buffer. A scrape was made through the confluent monolayer with a plastic pipette tip of 1mm diameter. Afterwards, the dishes were washed twice and incubated at 37°C in fresh DMEM containing 10% fetal bovine. At the bottom side of each dish, two arbitrary places were marked where the width of the wound was measured with the inverted microscope (objective x4). Motility was expressed as the mean ± SEM of the difference between the measurements at time zero and the 6 and 24h time period.

2.8 Matrigel invasion assay

The invasiveness of the breast cancer cells MDA-MB-231 stably transfected with SMARCAD1-shRNAs or control-shRNA was tested using BD Matrigel Invasion Chamber (8-μm pore size; BD Biosciences, Le Pont de Claix, France) according to manufacturer's protocol. Briefly, Cells (1 x 10^5 cells in 0.5 mL of media) were seeded into the upper chambers of the system, the bottom wells in the system were filled with DMEM supplemented with 10% fetal bovine serum as a chemo-attractant and then incubated at 37°C for 24h. Non-penetrating cells were removed from the upper surface of the filter with a cotton swab. Cells that have migrated through the Matrigel were fixed with 4% formaldehyde, stained with DAPI and counted in 25 random fields under a microscope. The assay was carried out in duplicate and repeated three times for quantitative analysis.

2.9 The Oris™ Collagen I Cell Invasion Assay

This experiment was used to investigate the impact of SMARCAD1 silencing on MDA-MB-231 cell invasion *in vitro* within a 3-dimensional extracellular matrix comprised of collagen type I from rat tail (AMS Biotechnology, Abingdon, UK). Cells were seeded at 100,000 cells / well and allowed to attach overnight onto coated plates. Once the cells formed a confluent monolayer, the silicone stoppers were removed and the cells were incubated for 48 h invasion. Cells were then labeled with Calcein AM (Invitrogen) and images were acquired, in the absence of the mask, by use of Olympus fluorescence microscope.

2.10 Cellular viability

MDA-MB-231-Control-shRNA, MDA-MB-231-Smarcad1-shRNA1 and MDA-MB-231-Smarcad1-shRNA3 cells were seeded in 96-well plates at a density of 5,000 cells / well. After 24h, cells were treated for 24h with increasing concentrations of frondoside A (0.01-5μM) or paclitaxel (1-64nM) in triplicate. Control cultures were treated with 0.1% DMSO. The effect of the drugs on cell viability was determined using a CellTiter-Glo luminescent cell viability assay (Promega Corporation, Madison, USA), based on quantification of ATP, which signals the presence of metabolically active cells. Luminescent signal was measured using GLOMAX Luminometer system. Data were presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which was assumed to be 100%.

2. 11 Statistical Analysis: In the above experiments, results were expressed as mean ± S.E.M. The difference between experimental and control values were assessed by ANOVA followed by Dunnett's post-hoc multiple comparison test. $P<0.05$ was taken to indicate a significant difference.

Chapter 3

Results

3.1 Human Breast Cancer Cell Lines Over-express SMARCAD1: The expression of the SMARCAD1 was initially investigated in the human breast epithelial cells NHME in comparison with several human breast cancer cell lines including ER-negative MDA-MB-231 and MDA-MB-231-1833 and ER-positive MCF-7 and T47D. Western blot analysis of crude protein lysates (100 μg) prepared from these cells was performed, using the mouse polyclonal antibody directed against SMARCAD1. We observe in Fig. 1 that all the cells exhibit mainly an immunoreactive band with a molecular weight of 117 KD, corresponding to the expected size of SMARCAD1. Together with this band, an additional minor band of 90KD was also detected in lysates from all the cells **(Fig 1).**

3.2 SMARCAD1 silencing in breast cancer cells MDA-MB-231: Cells were stably transfected with two different designs of SMARTvector 2.0 Lentiviral shRNA particles targeting SMARCAD1. Control cells were transfected with SMARTvector 2.0 Non-Targeting control particles. The positive clones (10 to 12 from each design) were selected under puromycin **(Fig 2A)** and analyzed using western blot to confirm specific SMARCAD1 silencing. The two different design of shRNA targeting SMARCAD1 induced 99% decreased in the SMARCAD1 protein level (SMARCAD1-shRNA1, and SMARCAD1-shRNA3). The selectivity of this silencing was confirmed by the fact that no impact on SMARCAD1 protein was observed in the cells transfected with shRNA control particles (control-shRNA) **(Fig 2B)**. The selected clones were GFP positive **(Fig 3).**

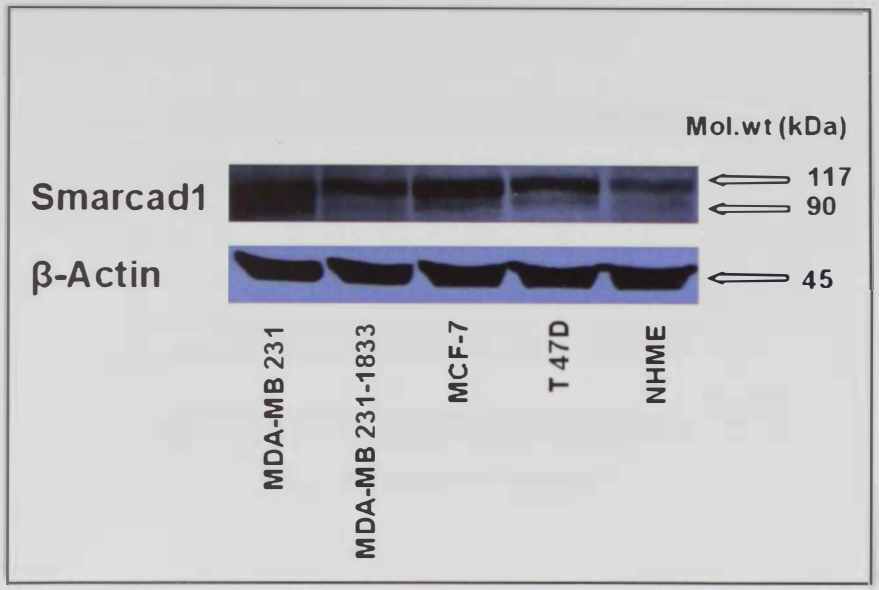
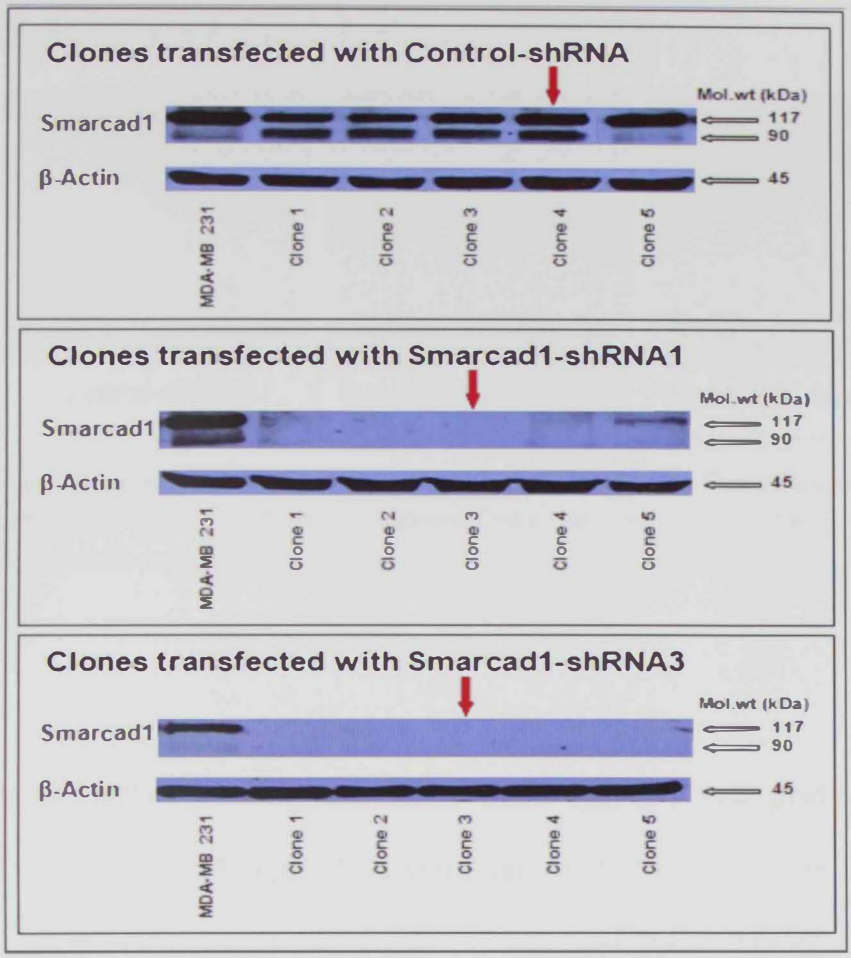


Figure 1: Western blots analysis of SMARCAD1 protein expression in human breast cancer cell lines MDA-MB-231, MDA-MB-231-1833, MCF-7 and T47D and in the normal human breast epithelial cells NHME.

A)



B)

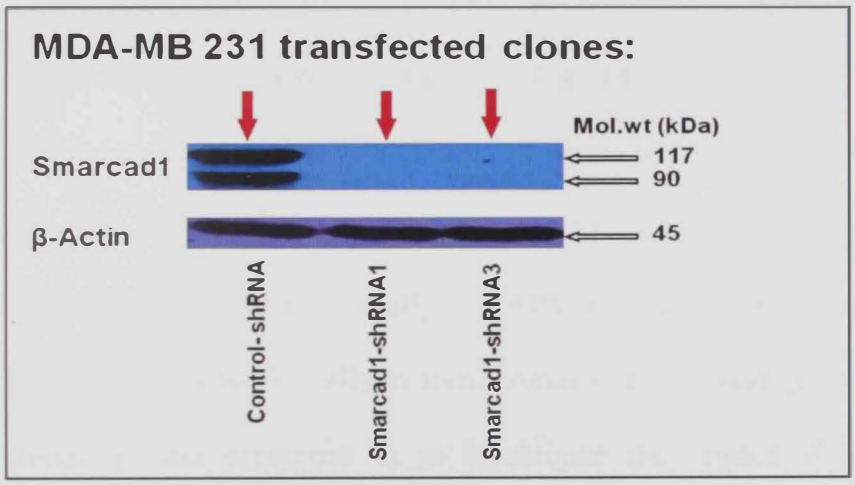


Figure 2: Western blots analysis of SMARCAD1 protein expression in: **A & B)** stably transfected MDA-MB-231-Control-shRNA (Clone 4), MDA-MB-231-SMARCAD1-shRNA1 (Clone 3), and MDA-MB-231-SMARCAD1-shRNA3 (Clone 3) cells.

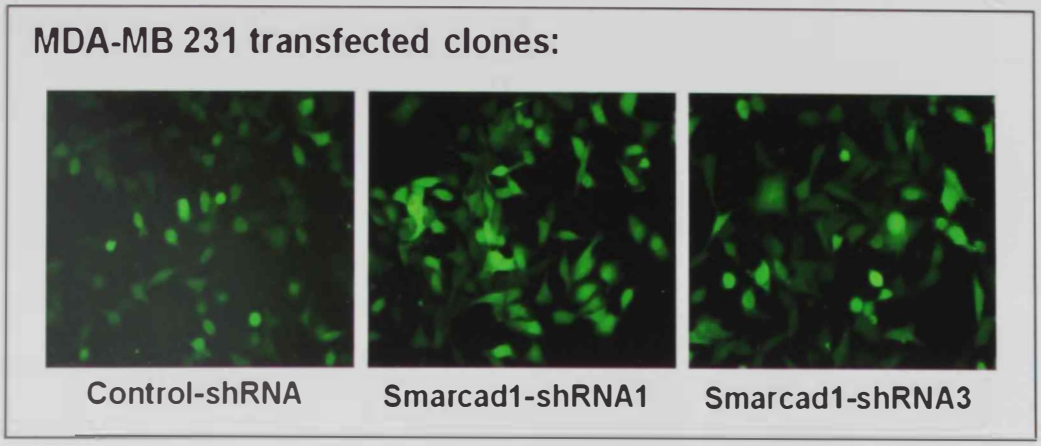


Figure 3: Immunofluorescence analysis of green fluorescence protein expression: the three selected clones from the control and the SMARCAD1 silenced cells were photographed for their GFP expression.

3.3 SMARCAD1 silencing decreased MDA-MB-231 cell proliferation and colonies growth in soft agar: To test the ability of SMARCAD1 to interfere with cancer cell proliferation, Control-shRNA cells and their SMARCAD1 silenced counterparts SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells were compared for their growth rates. As shown in **Fig. 4A**, SMARCAD1 silenced cells exhibit slower proliferation rates, as shown at days 3 to 5 in culture. At day 5, the inhibition was 55 and 35% respectively for SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells. Similarly, SMARCAD1 silencing strongly inhibited the ability of MDA-MB-231 cells to form colonies in soft-agar **(Figure 4B &C).** This interesting data prompted us to investigate the impact of SMARCAD1 silencing on the migration and invasion of MDA-MB-231 human breast cancer cells.

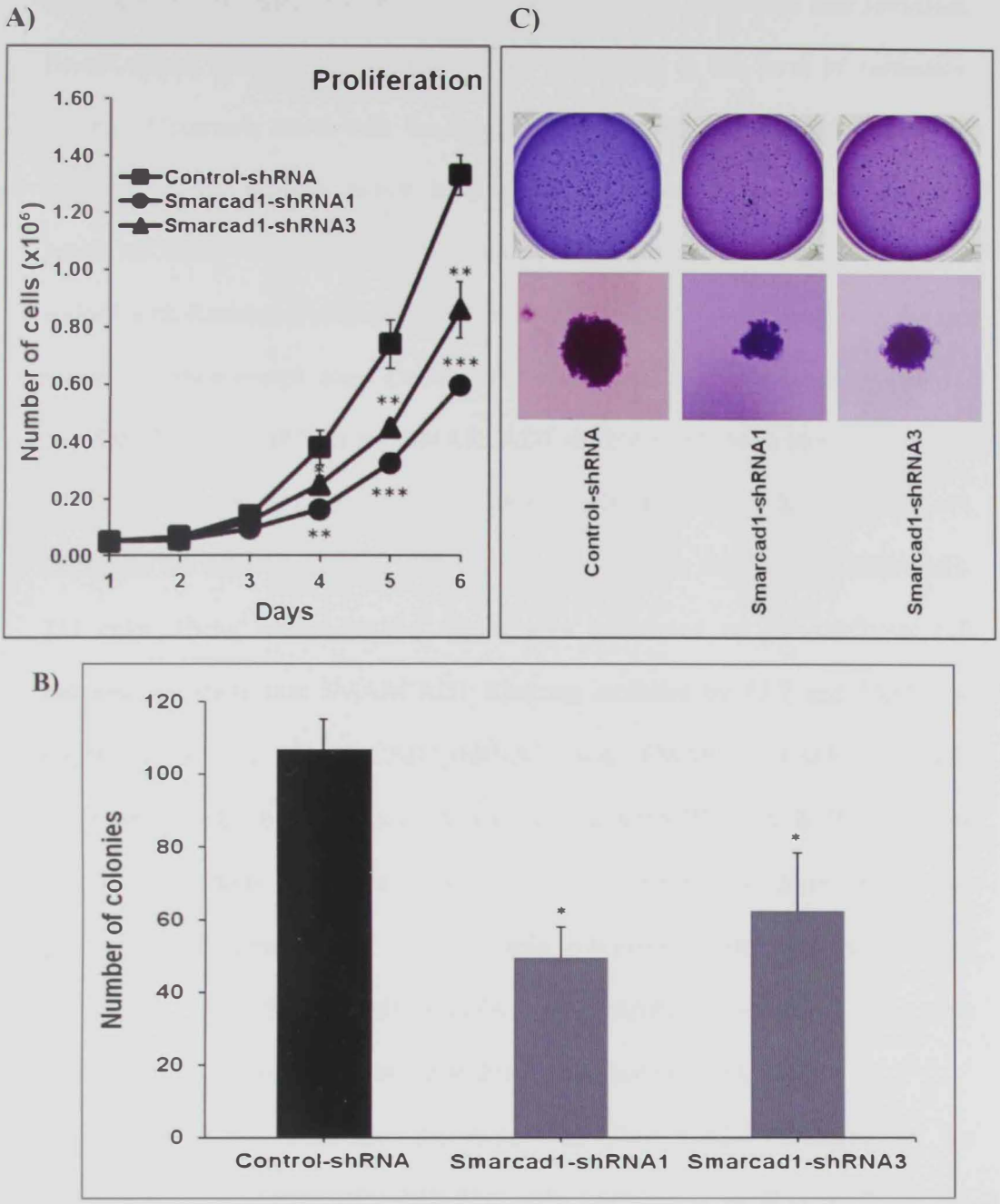


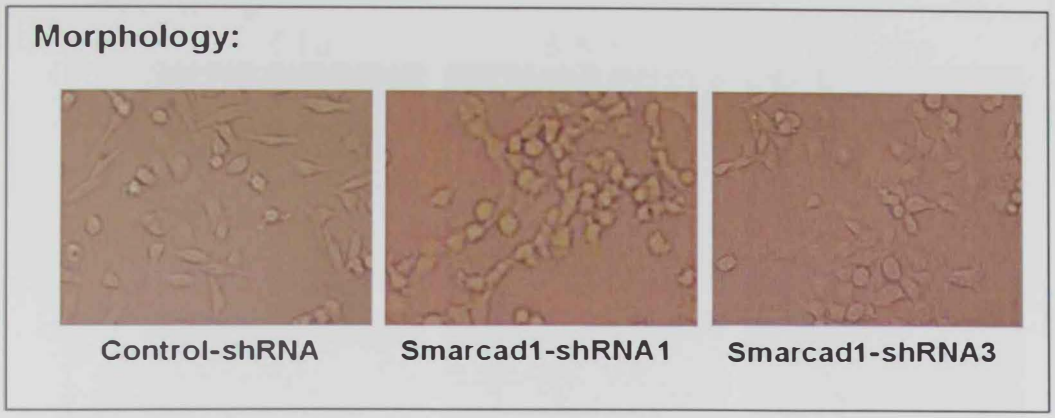
Figure 4: Impact of SMARCAD1 silencing on the proliferation and colonies growth of MDA-MB-231 breast cancer cells. A) Control and SMARCAD1 silenced cells were seeded into six-well tissue culture dishes (50,000 cells /dish) and counted daily for 5 days. Data are means ± SEM of 3 independent experiments. **B)** Inhibition of anchorage-independent colonies growth by stable silencing of SMARCAD1 in MDA-MB-231 cells. 20×10^3 of MDA-MB-231-Control-shRNA, MDA-MB-231-SMARCAD1-shRNA1, and MDA-MB-231-SMARCAD1-shRNA3 cells were plated in 0.3% soft agar. Three weeks later, colonies were stained with Giemsa and scored. Statistical differences obtained at *P<0.05, **P < 0.01, and ***P < 0.001. **C)** Pictures of the colonies formed in soft agar were photographed (top panel), and representative single colonies were enlarged 40 times (bottom panel).

3.4 SMARCAD1 silencing impairs breast cancer cell migration and invasion:

Breast cancer patients are at high risk of recurrence in the form of metastatic disease. Metastasis starts with the acquisition of a scattered phenotype and cell migration in the primary tumor, leading to local tissue invasion and entry into lymph or blood vessels. First, the actin cytoskeleton of these cancer cells was stained with rhodamine phalloidin and theirs nucleus was stained with DAPI for the analysis of their morphology. Contrast and fluorescent microscope analysis shows that SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells have stronger cell-cell adhesion in comparison with Control-shRNA cells **(Fig. 5A & B)**. Secondly, we examined the effect of SMARCAD1 silencing on cellular migration of MDA-MB-231 cells. Using wound-healing experiments performed on sub-confluent cell cultures, we show that SMARCAD1 silencing inhibited by 67.7 and 50.8% the migration of the SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells respectively at the 6h short incubation time considered **(Fig. 6A & B)** indicating that the inhibition of cellular migration is not due to the inhibition of cell proliferation. Control-shRNA cells were able to achieve a complete wound healing within 24h, while SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells were unable to fully colonize the wounds at this time period **(Fig. 6A & B).**

Finally, we examined the impact of SMARCAD1 silencing on the spontaneously invasive MDA-MB-231 cells. As shown in **Figure 7A & B,** selective and stable silencing of SMARCAD1 strongly reversed cellular invasiveness of MDA-MB-231 cells in matrigel matrix as well as in Oris invasion assay **(Figure 7A & B)**. All together, these results strongly suggest that SMARCAD1 may play an important role in breast cancer cell migration, invasion and metastasis.

A)



B)

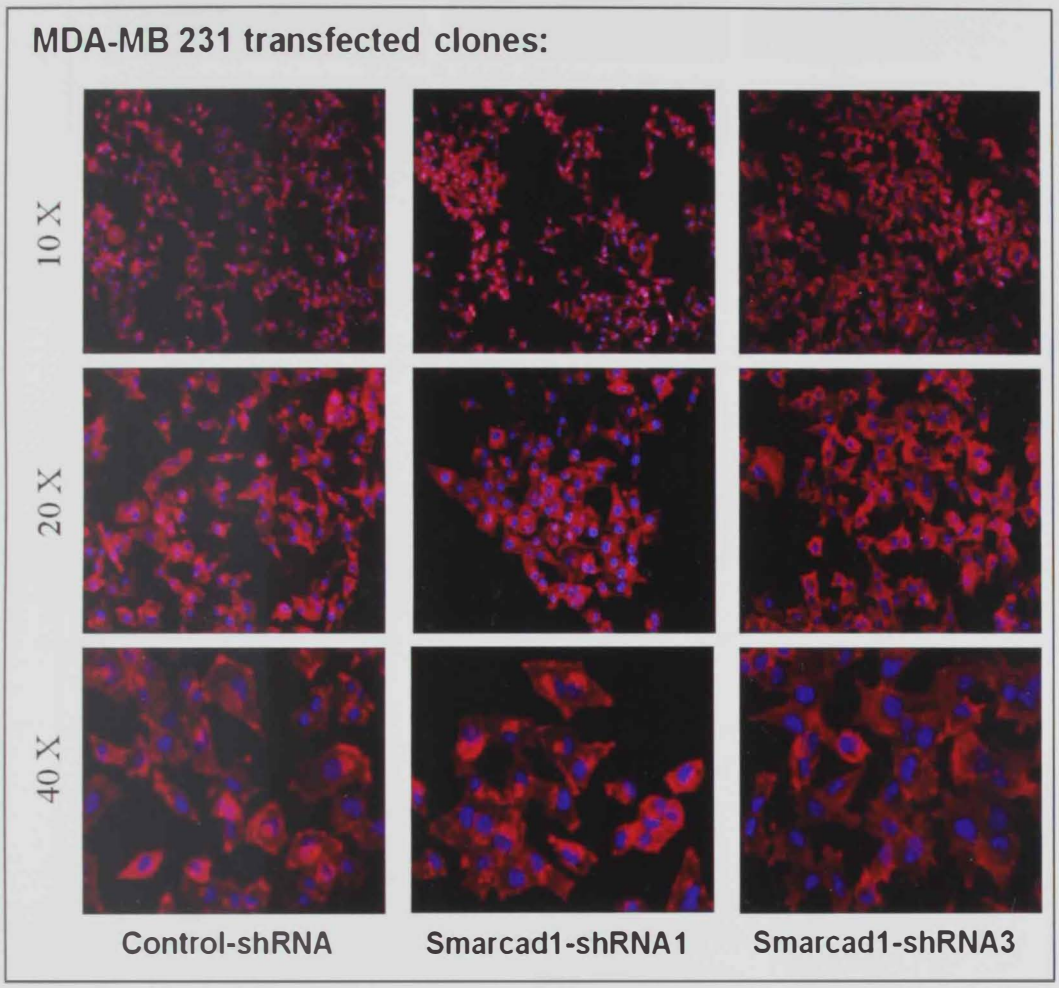
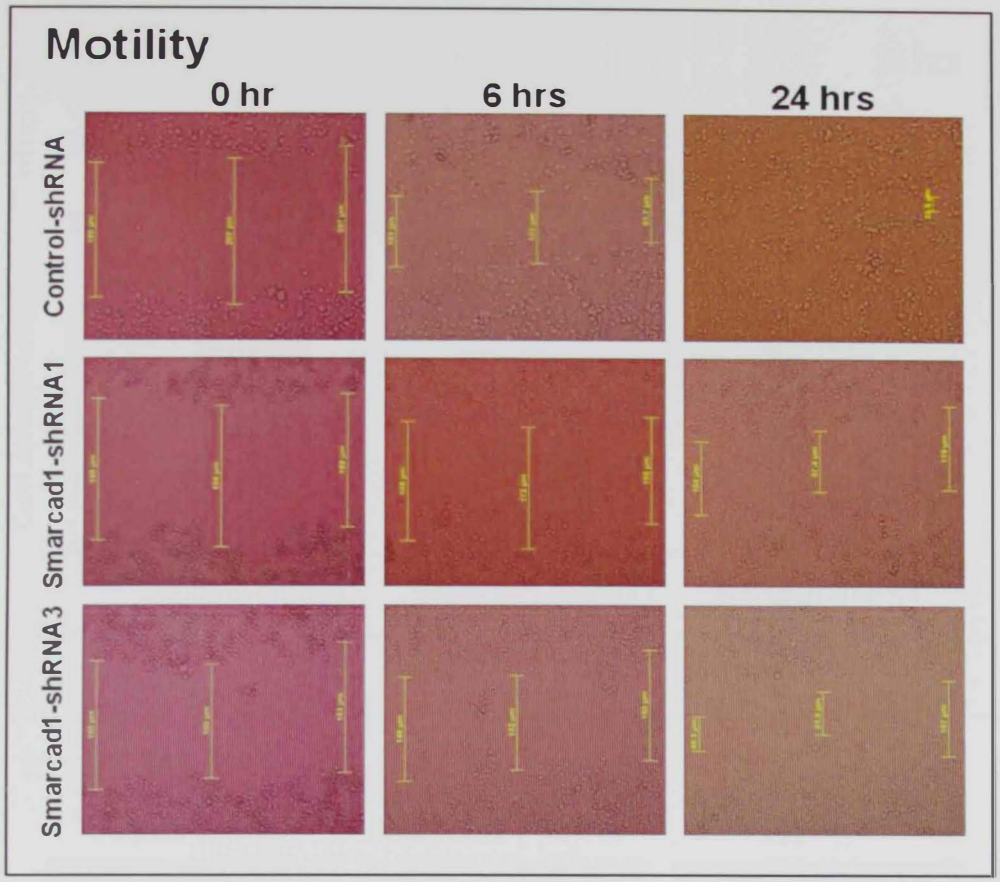


Figure 5: SMARCAD1 silencing impairs MDA-MB-231 breast cancer cell migration and invasion: Morphological changes observed in the SMARCAD1 silenced MDA-MB-231 cells were photographed using an inverted microscope **(A).** The actin cytoskeleton was then analyzed under a fluorescence microscope at three different magnifications after staining with the rhodamine phalloidin solution **(B).**

A)



B)

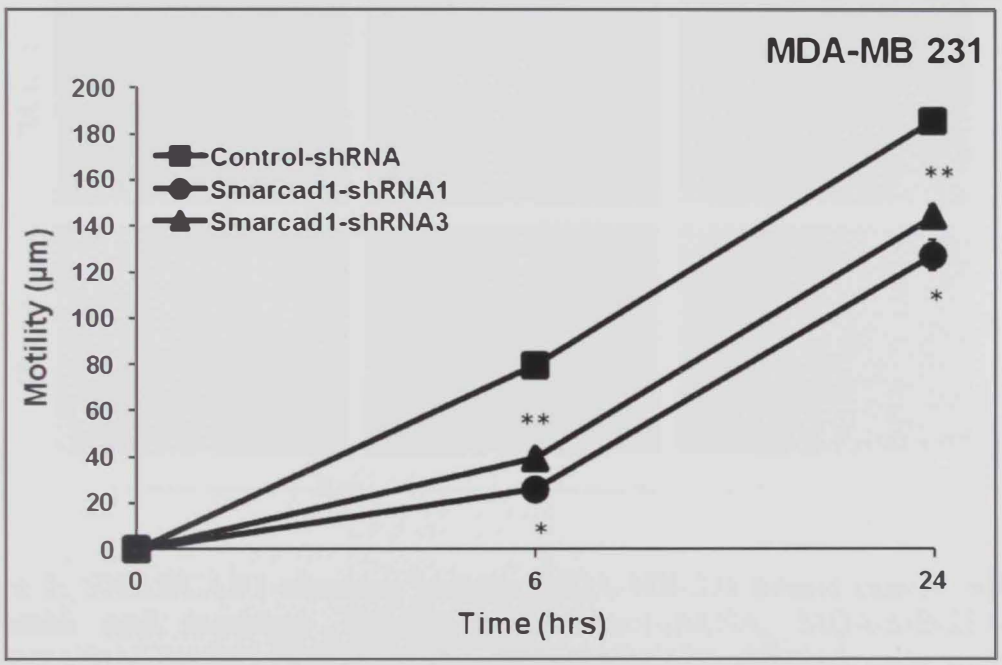
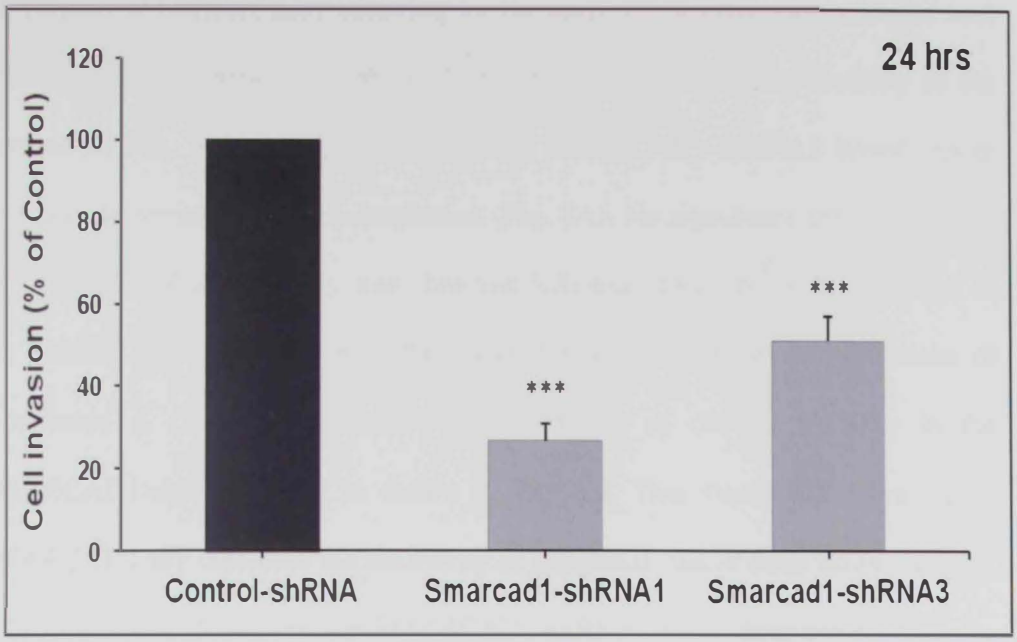


Figure 6: SMARCAD1 silencing impairs MDA-MB-231 breast cancer cell migration and invasion: Wounds were introduced in control and SMARCAD1 silenced confluent mono-layers cultured in the presence of serum. The mean distance that cells travelled from the edge of the scraped area for 6 and 24h at 37°C was measured in a blinded fashion, using an inverted microscope. Data are means ± SEM of four independent experiments **(A & B).**

A)



B)

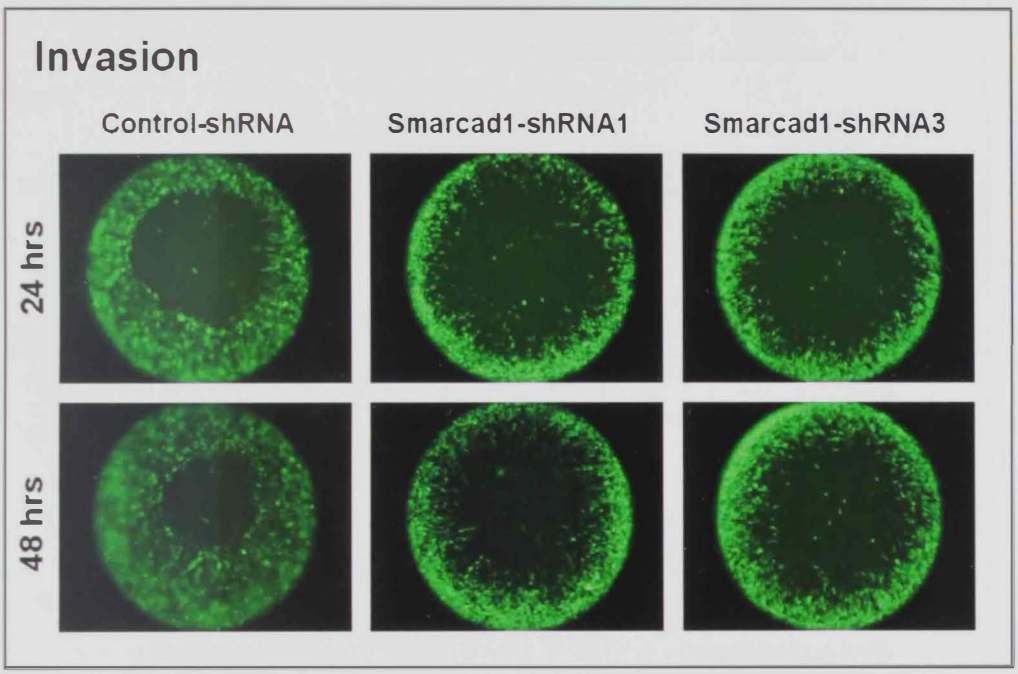


Figure 7: SMARCAD1 silencing impairs MDA-MB-231 breast cancer cell migration and invasion: MDA-MB-231-Control-shRNA, MDA-MB-231-SMARCAD1-shRNA1, and MDA-MB-231-SMARCAD1-shRNA3 cells were incubated for 24h in the presence of serum. Cells that invaded into Matrigel were scored as described in Materials and Methods **(A)**. Cells were incubated for 48h onto an Oris collagen type I cell invasion assay plate. Cell invasion images were acquired by fluorescence microscope **(B)**. All experiments were repeated at least three times. *Significantly different at $P < 0.05$, **Significantly different at $P < 0.01$, ***Significantly different at $P < 0.001$.

3.5 Impact of SMARCAD1 silencing on the anti-cancer effect of paclitaxel and Frondoside A *in vitro*: The effect of paclitaxel (1-64nM) on the viability of the Control-shRNA, SMARCAD1-shRNA1, and SMARCAD1-shRNA3 breast cancer cells was determined after 48h incubation **(Fig. 8A).** No significant enhancement of the inhibition of cell viability was observed following treatment with paclitaxel in the SMARCAD1 silenced cells **(Fig. 8A).** Interestingly, high concentrations of Frondoside A (2.5-5μM), enhances the inhibition of cellular viability in the SMARCAD1-shRNA1 cells as shown in **Fig. 8B.** This means that silencing of SMARCAD1 my increases the sensitivity of the breast cancer cells MDA-MB-231 to Frondoside A. However, the SMARCAD1-shRNA3 cells, does not enhance the sensitivity of the cells to Frondoside A.

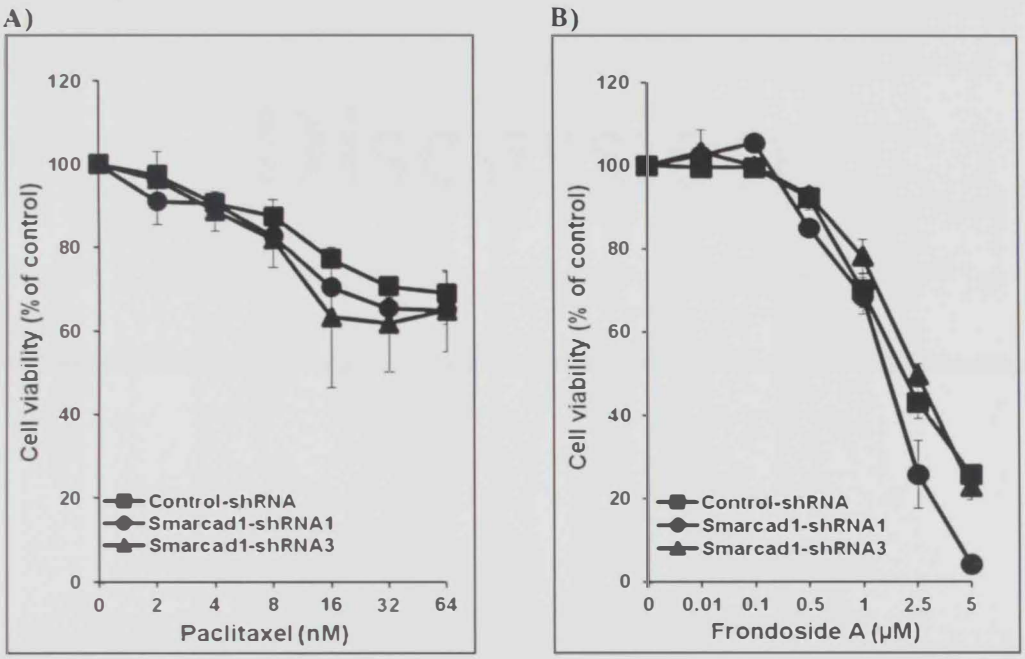


Figure 8: SMARCAD1 silencing enhances the inhibition of cellular viability induced by Frondoside A but not by paclitaxel. Control and SMARCAD1 silenced MDA-MB-231 cells were treated with vehicle (0.1% DMSO) and the indicated concentrations of paclitaxel **(A)** or Frondoside A **(B)** for 48h. Viable cells were assayed as described in Materials and Methods. All experiments were repeated at least three times. *Significantly different at $P < 0.05$, **Significantly different at $P < 0.01$, ***Significantly different at $P < 0.001$

Chapter 4

Discussion

Breast cancer is the most common cancer among woman, and it is responsible for 1.380.000 cases and 450.000 deaths each year worldwide [97]. Invasion leading to metastasis represents the most devastating attribute of cancer. Genes associated with invasion and metastatic virulence provides an aggressive edge in survival and proliferation during colonization of the metastatic site. Breast cancer patients are at high risk of recurrence in the form of metastatic disease.

The SMARCAD1 (SWI/SNF-related, Matrix-associated, Actin-dependent Regulator of Chromatin, containing DEAD/H box 1) protein is the human homolog of yeast Fun30 (Function Unknown Now 30) proteins. These proteins are members of the Snf2 helicase motif-containing protein families, and are known to act as ATP-dependent DNA transclocators. Previous studies have shown that yeast Fun30 deletions are resistant to ultraviolet (UV) radiation. The over-expression of Fun30 has been shown to affect chromosome stability, integrity, and segregation. Fun30 has also been shown to be a potential cyclin-dependent kinase (Cdk1)/Cdc28 substrate. The SMARCAD1 is a DEAD/H box-containing helicase, which includes proteins essential for replication, repair, and transcription. Expression analysis indicates that SMARCAD1 transcripts are ubiquitous, with particularly high levels in endocrine tissue. The gene for SMARCAD1 has been mapped to the chromosome 4q22-q23m, a region rich in break points and deletion mutants of genes involved in several human diseases, notably soft tissue leiomyosarcoma, hepatocellular carcinoma, and hematologic malignancies. Our laboratory have previously shown that the breast cancer cells ER-negative MDA-MB-231 and MDA-MB-231-1833 as well as ER-positive MCF-7 and T47D expressed a high level of SMARCAD1 in comparison with the normal breast epithelial cells NHME.

In this master research project, we investigated the impact of specific silencing of SMARCAD1 on human breast cancer cell proliferation, growth on soft agar, morphology, migration and invasion using human breast cancer cells estrogen receptors (ER)-negative MDA-MB-231. In this respect, the MDA-MB-231 cells were stably transfected with two different designs of SMARTvector 2.0 Lentiviral shRNA particles targeting SMARCAD1. Control cells were transfected with SMARTvector 2.0 Non-Targeting control particles. The positive clones (10 to 12 from each design) were selected under puromycin and the GFP positive clones were analyzed using western-blot to confirm specific SMARCAD1 silencing. The two different design of siRNA targeting SMARCAD1 induced 99% decreased in the SMARCAD1 protein level (SMARCAD1-shRNA1, and SMARCAD1-shRNA3). The selectivity of this silencing was confirmed by the fact that no impact on SMARCAD1 protein was observed in the cells transfected with shRNA control particles (control-shRNA). We demonstrated for the first time that silencing of SMARCAD1 resulted in a significant inhibition of cellular proliferation and colonies formation in soft agar, as well as cellular migration and invasion. All together, these results strongly suggest that SMARCAD1 may play an important role in breast cancer growth and metastasis.

It has been previously reported that deletion of Fun30, was associated with a delay in the cell cycle leading to an inhibition in the proliferation (Al Marzouqi et al, unpublished data). Similarly, we report in this study that silencing of SMARCAD1, the mammalian homologue of Fun30, is also associated with an inhibition of cell proliferation leading to a decrease in the colony growth.

Metastasis is the major cause of morbidity and mortality in breast cancer patients that causes nearly half a million deaths each year worldwide [98]. This

multi-step process involves increased motility of the tumor cells, invasion of nearby tissues and intravasation into blood and lymphatic circulation. Finally breast carcinomas colonize distant organs, mainly the brain, liver, bone and lungs. Patients with metastatic cancer have a low chance of achieving long-term survival after chemotherapy [99]. Metastasis can occur at later stages during therapy or several years following treatment.

Therefore, to understand the molecular mechanisms of cancer metastasis, it is necessary to identify the genes whose alterations accumulate during cancer progression as well as the genes whose expression is responsible for the acquisition of metastatic potential in cancer cells which can be classified into three groups: metastasis initiation, metastasis progression and metastasis virulence genes [100]. Initiation of metastatic genes involves promoting cell motility, invasion, epithelial-mesenchymal transition (EMT), extracellular matrix degradation, bone marrow progenitor mobilization and angiogenesis [101]. In this study, we observed from the morphology of SMARCAD1 silenced MDA-MB-231, that the silencing of SMARCAD1 is associated with stronger cell-cell adherence leading to the inhibition of cellular migration and invasion.

These results are in total agreement with recent work demonstrating that the silencing of BRG1, a core component of the SWI/SNF chromatin-remodeling complex, like the silencing of SMARCAD1 markedly inhibits breast cancer cell proliferation and reduces MMP-2, thereby inhibiting the ability of cells to migrate and to invade [102]. However, both studies are in contrast with previous publications reporting that chromosomal instability is associated with higher expression of genes implicated in Epithelial-Mesenchymal Transition, cancer invasiveness, and metastasis and with lower expression of genes involved in cell

cycle checkpoints, DNA repair, and chromatin maintenance [103], and that the alterations in chromatin play an important role in breast cancer progression and metastasis [104].

Frondoside A is a triterpenoid glycoside isolated from the Atlantic cucumber, *Cucumaria frondosa* [105]. Low concentrations of Frondoside A inhibit the growth and induced apoptosis of human pancreatic, leukemia, lung and breast cancer cells via caspase activation [106-109]. It has also been demonstrated that Frondoside A has an anti-invasive and anti-metastatic effect on human breast and lung cancer cells [108,109]. Frondoside A enhances breast cancer cell death induced by the chemotherapeutic agent Paclitaxel [109]. Paclitaxel has considerable clinical activity against a variety of malignancies including breast cancer. Paclitaxel, known to stabilize microtubules, inhibit cell division and induces death of breast cancer cells [110].

In the current study, we investigated the possible contribution of SMARCAD1 silencing to the anti-cancer effect of Paclitaxel and Frondoside A *in vitro*. No additional inhibition of cell viability was observed after treatment with Paclitaxel in the silenced SMARCAD1 cells in comparison with control-shRNA cells. However, the inhibition of cellular viability with high concentrations of Frondoside A was enhanced in the SMARCAD1-shRNA1 cells. This may suggest that we can increase the sensitivity of the breast cancer cell lines to Frondoside A by silencing SMARCAD1. This result is consistent with previous study showing that the loss of SMARCAD1 impairs end resection and recombinational DNA repair, and renders cells hypersensitive to DNA damage resulting from camptothecin or poly(ADP-ribose) polymerase inhibitor treatments [90].

In conclusion, we have identified that specific silencing of SMARCAD1 decrease cellular proliferation, colony growth, migration and invasion of the human breast cancer cells estrogen receptors (ER)-negative MDA-MB-231. This study is the first demonstration of the role of SMARCAD1 in human breast cancer progression. Increased SMARCAD1 expression may facilitate tumor progression by enhancing cell growth, migration and invasion. Our results imply that SMARCAD1 may serve as a prognostic marker as well as a potential therapeutic target for breast cancer.

Perspectives of the present study

1. Impact of SMARCAD silencing on trans-endothelial migration of MDA-MB-231 cells through HUVEC cells: Briefly, transwell filters will be coated with collagen type I and allowed to dry for 1 hour. HUVECs 150,000/well will then be seeded onto the rehydrated membrane and allowed to grow for 24 hours until a confluent monolayer is formed. Then, $1x10^5$ of the breast cancer cells MDA-MB-231 stably transfected with SMARCAD1-shRNAs or control-shRNA will then be loaded on top and incubated overnight in 0.1% serum, the bottom wells in the system will be filled with DMEM supplemented with 10% fetal bovine serum as a chemo-attractant. Non-penetrating cells in the upper chamber will removed with a cotton swab, whereas MDA-MB-231 in the bottom will be fixed with 4% formaldehyde, stained with DAPI and quantified for GPF expression in 25 random fields under a microscope [111].

2. Impact of SMARCAD silencing on the expression of the metastatic suppressor gene E-cadherin: It is well known that disruption of cell-cell adhesion during cancer progression is the initial stage required for the acquisition of invasive properties and characterized by a decreased expression of E-cadherin. E-cadherin, a calcium-dependent, cell adhesion molecule, is considered as a tumor suppressor in breast cancer [112]. Decrease of E-cadherin expression is a critical and necessary event required in the disruption of cell-cell adhesion and thus for the acquisition of invasive phenotype of various tumors including breast cancer. In fact, downregulation or loss of E-cadherin during cancer progression is associated with aggressive behavior of the tumor and poor prognosis [113]. Conversely, expression of E-cadherin led to a reduced progression and invasion of breast cancer cells [114]. In this context, we will investigate the impact of SMARCAD silencing on the expression of the metastatic suppressor gene E-cadherin.

3. Impact of SMARCAD1 silencing on cellular aggregation: We showed in this thesis that SMARCAD1-shRNA1 and SMARCAD1-shRNA3 cells have stronger cell-cell adhesion compared to control-shRNA cells. Therefore we would like to investigate the impact of SMARCAD1 silencing on the MDA-MB-231 adhesive properties using two assays.

A. Slow aggregation assay: single-cell suspensions will be seeded onto a semi-solid agar medium. After 24 h, aggregate formation will be evaluated subjectively by phase-contrast microscopy.

B. Fast aggregation assay: single-cell suspensions will be prepared using an E-cadherin saving procedure [115]. Cells will be incubated in an isotonic buffer containing 1.25mM Ca^{2+} under gyratory shaking for 30 min at 37C. Particle

diameters will be measured in a particle size counter (LS 200; Beckman Coulter, Miami, Fl, US) at the start (t0) and after 30-min incubation (t30), and plotted against percentage volume distribution.

4. Ability of SMARCAD1 to transactivate different subsets of target genes to orchestrate cell proliferation and colony growth or migration and invasion: This objective will be investigated in MDA-MB-231 cells transfected with SMARCAD1-shRNAs (SMARCAD1-shRNA1 & 3) or control sequences (Control-shRNA) using real-time PCR Array of Human Signalling in 384-well Plate containing 380 human genes associated with breast cancer, transcription, survival/apoptosis, cell cycle, Epithelial Mesenchymal transition, invasion, cell-cell adhesion, cell-matrix adhesion, metastasis, DNA replication/repair, and nucleotide synthesis and 4 assays of endogenous control gene (GAPDH) in a customize array (Applied Biosystems, US). Genes that are more than two fold up or down regulated will be analysed using RT-PCR or Western-blot.

5. Impact of SMARCAD1 silencing on tumor growth and metastasis in nude mice

A. Tumor growth assay *in vivo*: Six-week-old athymic NMRI nude mice (nu/nu, Charles River, Suizfeld, Germany) will be maintained under specified pathogen-free conditions. Human breast cancer cells MDA-MB-231 stably transfected with SMARCAD1-shRNAs (SMARCAD1-shRNA1, and SMARCAD1-shRNA3) or control sequences (Control-shRNA) (5×10^6 cells mixed v/v with matrigel) will be injected subcutaneously into the lateral flank of the mice. Throughout this study, nude mice will be housed in filtered-air laminar

flow cabinets and manipulated following aseptic conditions. Procedures involving animals and their care will be conducted in conformity with Institutional guidelines that are in compliance with college of Medicine & Health Sciences, national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; and NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). Tumor dimensions will be measured with calipers every week. Tumor volumes (V) were calculated using the formula: V = a x b2 x 0.4, with "a" being the length and "b" the width of the tumor. The animals will be sacrificed six weeks after cells injection and tumors and axillary lymph nodes will be excised and weighed to determine the impact of SMARCAD1 silencing on tumor growth and lymph node metastasis.

B. Metastasis assay *in vivo*: Six-week-old athymic NMRI nude mice (nu/nu, Charles River, Suizfeld, Germany) will be maintained under specified pathogen-free conditions. Human breast cancer cells MDA-MB-231 stably transfected with SMARCAD1-shRNAs (SMARCAD1-shRNA1, and SMARCAD1-shRNA3) or control sequences (Control-shRNA) (1 x 10^5 cells in 100µl media) will be injected intravenously into the lateral tail vein of the mice. Throughout this study, nude mice will be housed in filtered-air laminar flow cabinets and manipulated following aseptic conditions. Procedures involving animals and their care will be conducted in conformity with Institutional guidelines that are in compliance with college of Medicine & Health Sciences, national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; and NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). The animals will be sacrificed three months after

cells injection and lungs will be excised and weighed to determine the impact of SMARCAD1 silencing on lung metastasis. These cells are stably transfected with a vector encoding green fluorescence protein, to allow a rapid and reliable quantification of micro-metastases in the lungs.

6. Immunohistochemical expression of SMARCAD1 in normal and breast cancer tissues: We have a total of 58 breast samples of breast carcinomas formalin-fixed paraffin-embedded retrieved from the Department of Pathology archives that will be used after receiving ethical approval from the Al Ain Medical District Human Research Ethics Committee. These cases have been previously studied and characterized for their ER, PR and HER2 status. We also have 41 tumor-free tissues from same group of patients. Immunostaining of SMARCAD1 will be carried out on deparaffinized sections using the avidin-biotin-peroxidase complex method. After inhibition of endogenous peroxidases with 3% hydrogen peroxide, slides will be washed in PBS and incubated with 10% BSA at room temperature for 60 minutes. SMARCAD1 immunoreactivity will be detected using a goat polyclonal antibody directed against the human SMARCAD1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature in a humidified chamber. SMARCAD1 immunohistochemistry specificity will be checked by omission of primary antibody. All slides will be rinsed three times with TBS; sections will be incubated with biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA) for 60 minutes at room temperature. The antigen-antibody complex will be revealed with avidin-biotin-peroxidase complex for 60 minutes according to the manufacturer's instructions for the Vectastain ABC kit (Vector Laboratories). Staining will done for 5 minutes with 3,3¶-diaminobenzidine (Sigma-Aldrich, Lyon, France). All slides will be counterstained

with hematoxylin. A semi-quantitative estimation of the number of positive cells will be done by counting 1,000 reactive and nonreactive cells in 10 successive fields at the original 100 X magnification.

Chapter 5

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على نوع الجين الصامت SMARCAD1. التصميمان المختلفان لي (shRNA) وهو عبارة عن سلسة قصير للحمض النووي الريبي والذي يستخدم للإسكات الجينات, ويستهدف SMARCAD1 والذي يحث على إنقاص بنسبة 99% في مستوى بروتينات SMARCAD1 في التصميمان المختلفان (SMARCAD1-shRNA1) و (SMARCAD1-shRNA3). تأكيد إنتقاء لهذا النوع من إسكات الجينات ليس به اي تأثير على بروتينات SMARCAD1، وهذا لوحظ في الخلايا التي تم التأثير عليها بي الحمض النووي الريبي (control-shRNA).

نحن أثبتنا للأول مرة أن إسكات الجين SMARCAD1 يؤدي إلى تنشيط هام وفعال لتكاثر الخلايا، تجمع و تشكيل الخلايا على شكل مستعمرات في الأجار اللين، هجرة الخلايا و غزوها للأعضاء مختلفة من الجسم. كل ذلك معا، هذه النتائج تقدم و بقوة أن SMARCAD1 يلعب دورا أساسيا مهما في تكاثر خلايا سرطان الثدي و إنتشاره.

إن تحديد أنواع جديدة من المستجيبات الجزيئية و مسارات الإشارات للخلايا والتي تعني بقدرة خلايا الثدي السرطانية للتكاثر، الإنتشار و غزو مناطق سليمة من الجسم والذي يؤدي الى فعالية, و إستهداف علاجات مختلفة وفعالة لسرطان الثدي.

ملخص البحث

من أكثر انواع السرطان إنتشارا بين النساء و الأكثر شيوعا لعودة المرض هو سرطان الثدي, وهناك أكثر من مليون حالة وفاة سنويا بسببه. الموروثة المسببة للمرض في مرحلة تكوين المستعمرات، تهيء الخلايا للتكاثر بسرعة كبيرة جدا و الإنتشار في أعضاء حيوية و الإستقرار هناك و من ثم التكاثر مجداد. إن SMARCAD1 (SWI/SNF) تربط الخلايا ببعض وأن منظم الكروماتين المعتمد على الأكتين هو النموذج البشري المشابه لبروتين الخميرة Fun30 غير محدد الوظيفة بعد, هذه البروتينات هي أفراد Snf2 ضمن عائلة البروتينات المرسومة والمعروف عنها انها تعمل مع ATP و ناقلة النوكليوتيديل الدناوية لي DNA. و قد أظهرت الدراسات السابقة, أن خمول بروتين الخمائر Fun30 يقاوم الأشعة فوق البنفسجية و أن زيادة نسبة FUN30 يؤثر على الإستقرار بين الكروموسومات, و تكاملها، و تخصصها، كما أظهر بروتين Fun30 أنه يمكن أن يعمل مع الإنزيم Cyclin-dependent kinase (Cdk1) /Cdc28.

إن SMARCAD1 هو DEAD/H box يحتوي على Helicase والذي يتضمن البروتينات اللازمة لعملية المضاعفة (الإستنساخ) و الإصلاح، و بالإضافة لما ذكر فإن SMARCAD1 لديه إشارات من النواة و مواقع أخرى تحدد الترابط بين البروتينات (بروتين-بروتين). إن التحليل يدل على أن نسخ SMARCAD1 المنتشر في كل مكان و خصوصا في نسيج الغدد الصماء حيث يكون مستواها عالي. و الموروثة المسؤولة عن SMARCAD1 وجدت في الكروموسوم 4q22-q22m وهو موقع غني بنقاط التكسر و الطفرات للموروثات المرتبطة بعدد من الأمراض البشرية مثل: النسيج الرخو لساركومة العضلة الملساء, سرطان الخلايا الكبد, و سرطان خلايا الدم.

وقد أفيد مؤخرا, أن موقع الإرتباط الداخلي لي SMARCAD1/KIAA112 مجاورة لمواقع بدء النسخ. إضافة إلى ذالك لوحظ أن في الخلايا البشرية حيث SMARCAD1 يوجد في جينات الخلايا E1A-expressing, تمكنه من زيادة سرعة النشاط من خلال ترتيب الموروثات التي يمكن SMARCAD1 بلعب دورا في إستقرارية ة تطور هذه الموروثات.

ظهر مسبقا في مختبرنا, أن مستقبلات الإستروجين في خلايا الثدي السرطانية من نوع MDA-MB-231 (ER)-negative و (ER)-positive) و MXF-7 و T47D يظهر مستوى مرتفع جدا من SMARCAD1 بالمقارنة عن مثيلاتها من خلايا نسيج الثدي الطبيعي NHME.

في بحث دراسة الماجستير، جرى التحقيق في تأثير إسكات نوع محددة من SMARCAD1 في الخلايا البشرية لسرطان الثدي لتحقق من سرعة تاكثر الخلايا, و النمو في الأجار اللين, و تشكيل الخلايا, وهجرة الخلايا و غزوها للأعضاء أخرى بإستخدام خلايا سرطان الثدي التي تكون لا علاقة لها بمستقبلات الإستروجين.

في هذا السياق, تم التأثير على خلايا سرطان الثدي بتصميم نوعين, وهما: SMARTvector 2.0 Lentiviral والتي تستهدف SMARCAD1. الخلايا الرئيسة تم التأثير عليها بي SMART vector 2.0 التي لا تستهدف الخلايا الرئيسة. تم إختيار الإستنساخ الإيجابي من (10-12) من كل تصميم. و أختيرت بوجود مضاد حيوي وهو بوروميسين و البروتين الأخضر المتالق (GFP positive clones) ثم تم تحليل هذه الخلايا الإيجابية المتجمعة باستخدان تقنية طريقة لطخة ويسترن للتأكيد

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